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De Rosa, A ; Tirino, V ; Paino, F ; Tartaglione, A ; Mitsiadis, T A ; Feki, A ; d'Aquino, R ; Laino, L ; Colacurci, N ; Papaccio, G

Abstract: Mesenchymal stem cells are present in many tissues of the human body, including amniotic fluid (AF) and dental pulp (DP). Stem cells of both AF and DP give rise to a variety of differentiated cells. In our experience, DP stem cells (DPSCs) display a high capacity to produce bone. Therefore, our aim was to investigate if AF-derived stem cells (AFSCs) were able to undergo bone differentiation in the presence of DPSCs. AFSCs were seeded under three different conditions: (i) cocultured with DPSCs previously differentiated into osteoblasts; (ii) cultured in the conditioned medium of osteoblast-differentiated DPSCs; (iii) cultured in the osteogenic medium supplemented with vascular endothelial growth factor and bone morphogenetic protein-2 (BMP-2). Results showed that AFSCs were positive for mesenchymal markers, and expressed high levels of Tra1-60, Tra1-80, BMPR1, BMPR2, and BMP-2. In contrast, AFSCs were negative for epithelial and hematopoietic/endothelial markers. When AFSCs were cocultured with DPSCs-derived osteoblasts, they differentiated into osteoblasts. A similar effect was observed when AFSCs were cultured in the presence of a conditioned medium originated from DPSCs. We found that osteoblasts derived from DPSCs released large amounts of BMP-2 and vascular endothelial growth factor into the culture medium and that those morphogens significantly upregulate RUNX-2 gene, stimulating osteogenesis. This study highlights the mechanisms of osteogenesis and strongly suggests that the combination of AFSCs with DPSCs may provide a rich source of soluble proteins useful for bone engineering purposes.

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Amniotic Fluid-Derived Mesenchymal Stem Cells Lead to Bone Differentiation when Cocultured with Dental Pulp Stem Cells

Alfredo De Rosa, D.D.S.,[1,*] Virginia Tirino, B.Sc., Ph.D.,[2,*] Francesca Paino, Ph.D.,[2] Antonella Tartaglione, M.D.,[3] Thimios Mitsiadis, D.D.S., Ph.D.,[4] Anis Feki, M.D., Ph.D.,[5] Riccardo d'Aquino, D.D.S.,[1,2] Luigi Laino, D.D.S.,[1] Nicola Colacurci, M.D.,[3] and Gianpaolo Papaccio, M.D., Ph.D.[2]

Mesenchymal stem cells are present in many tissues of the human body, including amniotic fluid (AF) and dental pulp (DP). Stem cells of both AF and DP give rise to a variety of differentiated cells. In our experience, DP stem cells (DPSCs) display a high capacity to produce bone. Therefore, our aim was to investigate if AF-derived stem cells (AFSCs) were able to undergo bone differentiation in the presence of DPSCs. AFSCs were seeded under three different conditions: (i) cocultured with DPSCs previously differentiated into osteoblasts; (ii) cultured in the conditioned medium of osteoblast-differentiated DPSCs; (iii) cultured in the osteogenic medium supplemented with vascular endothelial growth factor and bone morphogenetic protein-2 (BMP-2). Results showed that AFSCs were positive for mesenchymal markers, and expressed high levels of Tra1-60, Tra1-80, BMPR1, BMPR2, and BMP-2. In contrast, AFSCs were negative for epithelial and hematopoietic/endothelial markers. When AFSCs were cocultured with DPSCs-derived osteoblasts, they differentiated into osteoblasts. A similar effect was observed when AFSCs were cultured in the presence of a conditioned medium originated from DPSCs. We found that osteoblasts derived from DPSCs released large amounts of BMP-2 and vascular endothelial growth factor into the culture medium and that those morphogens significantly upregulate RUNX-2 gene, stimulating osteogenesis. This study highlights the mechanisms of osteogenesis and strongly suggests that the combination of AFSCs with DPSCs may provide a rich source of soluble proteins useful for bone engineering purposes.

Introduction

Human mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into several lineages, including bone, cartilage, muscle, tendon, ligament, fat, and a variety of other connective tissues.[1,2] Morphologically, undifferentiated MSCs are spindle shaped and resemble fibroblasts. MSCs have been mostly identified in adult bone marrow; recent reports have shown that MSCs are also present in both adult and fetal peripheral blood, fetal liver, fetal spleen, placenta, umbilical cord,[3–5] Wharton's Jelly, dental pulp (DP),[6–8] and amniotic fluid (AF).[9–11]

Lately, some reports evidenced that human AF contains a variety of stem cells that are shed from embryonic and extraembryonic tissues during fetal development. Epithelial cells derived from human AF can be transformed into neurons, astrocytes, and oligodenrocytes, and these cells have been indicated as possible candidates for transplantation therapy of neurodegenerative diseases.[12] Further, cells expressing the stem cell marker Oct-4 were also found to be present in human AF. Due to this relevant feature, AF has been proposed as an appealing source of multipotent stem cells that can be used without the ethics concerns associated with human embryonic stem cells.[13] Therefore, AF might be a potentially ideal source of MSCs for therapeutic transplantation.[14]

In previous studies we have demonstrated that MSCs isolated from DP (DPSCs) coexpress the CD34 and CD117 markers,[6–8,15,16] proliferate extensively, have a long lifespan,

[1]Dipartimento di Discipline Odontostomatologiche, Ortodontiche e Chirurgiche, Secondo Ateneo di Napoli, Napoli, Italy.
[2]Dipartimento di Medicina Sperimentale, Sezione di Istologia ed Embriologia, Secondo Ateneo di Napoli, Napoli, Italy.
[3]Dipartimento di Ginecologia ed Ostetricia, Secondo Ateneo di Napoli, Napoli, Italy.
[4]Department of Orofacial Development and Structure, Institute of Oral Biology, ZZMK, Faculty of Medicine, University of Zurich, Zurich, Switzerland.
[5]Department of Obstetrics and Gynecology, Geneva University Hospitals, Geneva, Switzerland.
*These authors equally contributed to the study.

and maintain their multipotential capabilities for many generations. In addition, DPSCs are capable of differentiating into adipocytes, chondrocytes, and myocytes when cultured with the adipo-condro-myogenic medium, and in osteoblasts and bone when cultured in the standard medium supplemented with 20% fetal bovine serum (FBS) and without addition of specific osteogenic morphogens.[6–8]

Taking all the above into consideration, our aim was to detect MSCs from AF and then to challenge them with DPSCs to ascertain whether they could differentiate into a bone tissue.

Materials and Methods

Culture of AF-derived MSCs

Amniotic fluid samples ($n = 20$, 20 mL each) were obtained by amniocentesis performed between 16 and 20 weeks of gestation during routine prenatal diagnoses. Clinical indications for amniocentesis were the advanced maternal age, the familiar or personal history of birth defects, and the risk that the fetus might have a chromosome abnormality or an inherited condition. The cytogenetic analyses revealed normal karyotypes for all donors. Cells were isolated from the AF samples no more than 4 h before being used. The samples were centrifuged at 1300 rpm for 5 min, and all the isolated cells were plated in 25 cm^2 flasks containing low-glucose Dulbecco's modified Eagle's medium (Invitrogen, Milan, Italy) supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, 10 ng/mL basic fibroblast growth factor, 10 ng/mL epidermal growth factor (all from Peprotech, Rocky Hill, NJ), and 20% FBS (Invitrogen). The medium was renewed after the cells were incubated at 37°C with 5% humidified carbon dioxide for 7 days, and the nonadherent AF cells were removed. The medium was then replaced twice weekly until the cells reached 70% confluence, when they were treated with 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (Invitrogen) for 3 min. The AF-derived stem cells (AFSCs) were collected and replated in a split ratio of 1:3 under the same culture conditions.

Culture of DPSCs and osteogenic differentiation

Human DPs were extracted from adult teeth of healthy subjects aged 21–45 years, according to our protocol.[6] Briefly, the pulp was gently removed and immersed in a digestive solution: 3 mg/mL type I collagenase plus 4 mg/mL dispase in phosphate-buffered saline (PBS) for 1 h at 37°C. Once digested, cells were immersed in the α-MEM culture medium supplemented with 20% FBS, 100 μM 2P-ascorbic acid, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all purchased from Invitrogen).

At day 22 of culture, at least 1,000,000 cells per sample were detached from the flasks and sorted for CD117, CD34, and STRO-1 to obtain DPSCs. Sorted cells were cultured in α-MEM with 20% FBS. When DPSCs showed signs of differentiation, usually by day 30 as previously reported,[6–8] cells were examined under flow cytometry with the following mouse anti-human antibodies: osteocalcin, CD44, and the transcription factor RUNX-2 (all from Santa Cruz, Santa Cruz, CA). For RUNX-2 intracellular analysis, cells were stained with Caltag Fix & Perm Kit (Invitrogen) according to the manufacturer's instructions. CD44 and osteocalcin antibodies were incubated for 30 min at 4°C in the dark. After incubation, cells were rinsed and analyzed by flow cytometry. DPSCs, differentiated into osteoblasts and positive for CD44, osteocalcin, and RUNX-2, were used for the coculture experiments described below.

Cocultures of AFSCs and $OC^+RUNX\text{-}2^+$ DPSCs

At day 15 of culture, after cytometric analysis, samples of 100,000 AFSCs were plated in 16 mm wells and cocultured with 50,000 OC/RUNX-2-positive DPSCs, using 0.4 μm cell culture inserts (Falcon-BD, Le Point de Clex, France). As controls, MSCs were cultured with the osteogenic medium, osteogenic medium supplemented with 10 ng/mL vascular endothelial growth factor (VEGF) (Sigma–Aldrich, Milan, Italy), and 20 ng/mL bone morphogenetic protein-2 (BMP-2) (Sigma–Aldrich) and with the standard medium. Plated cells were incubated at 5% CO_2 for 30 days. The culture medium contained 10 μg/mL α-ascorbic acid, 10 mM β-glycerophosphate, 100 nM dexamethasone, and gentamicin. The medium was changed twice a week.

Stimulation of AFSCs with the conditioned medium of $OC^+RUNX\text{-}2^+$ DPSCs

At day 15 of culture, after cytometric analyses, samples of 100,000 AFSCs were plated in 16 mm wells and stimulated with the conditioned medium (CM) prepared as follows: after 3 days of culture with 1,000,000 $OC^+RUNX\text{-}2^+$ DPSCs, the supernatant was collected and filtered through a 0.22 μm strainer. AFSCs were then cultured with the CM for 15 and 30 days, changing the CM twice a week. Control samples were cultured with the standard medium.

BMP-2 and VEGF enzyme-linked immunosorbent assay analyses

To evaluate BMP-2 and VEGF levels in the CM, supernatant was collected from cultures at 24, 48, 72, and 96 h from plating $OC^+RUNX\text{-}2^+$ DPSCs. CM aliquots were stored at −20°C. After thawing at room temperature, 0.5 mL was collected from aliquots and analyzed with an ELISA kit for BMP-2 and VEGF (R&D, Milan, Italy).

Immunofluorescence

AFSCs cultured in 24-well plates were fixed in 4% paraphormaldeyde/1% Triton in PBS for 30 min at 4°C, washed in PBS, treated with 5% milk for 60 min at room temperature, and then stained with primary antibodies at 4°C over night. The primary antibodies were the following: BMPR1, BMPR2, BMP-2, CD29, CD34, CD44, CD90, CD117, CD133, OCT3/4, SSEA-4, Tra1-60, Tra1-80, stemness markers, and osteocalcin as an osteogenic marker. The secondary antibodies were FITC- and PE-conjugated mouse antigoat (Santa Cruz) incubated for 60 min at 4°C. The nuclei were stained with 4′, 6-diamidino-2-phenylindole. Cells were then washed and observed under the fluorescence microscope (Nikon TE 2000-S, Milan, Italy). Isotypes and nonprobed cells were used as controls.

Flow cytometry

After 15 days of culture in the standard medium, AFSCs were analyzed by flow cytometry for the following antibodies:

PE-conjugated mouse anti-human CD29, FITC-conjugated mouse anti-human CD31, FITC-conjugated mouse anti-human CD34, FITC-conjugated mouse anti-human CD44, FITC-conjugated mouse anti-human CD45, PE-conjugated mouse anti-human CD56, FITC-conjugated mouse anti-human CD90, PE-conjugated mouse anti-human CD133, and PE-conjugated mouse anti-human CD326. All antibodies were purchased from BD Pharmingen (Buccinasco, Milan, Italy) except CD133, which was purchased from Miltenyi Biotec (Calderara di Reno, Bologna, Italy). The antibodies were incubated for 30 min at 4°C in the dark. After incubation, the cells were washed and then analyzed by flow cytometry at a FACSVantage with CellQuest software (Becton & Dickinson, Mountain View, CA).

RT-polymerase chain reaction

Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. RNA concentration and purity were determined using an ultraviolet spectrophotometer by A_{260} and A_{260}/A_{280} ratio, respectively. The integrity of total RNA was assessed on standard 1% agarose/formaldehyde gels. The RNA samples were treated with DNase I to remove residual traces of DNA. cDNA was obtained from 1 μg of total RNA, using reverse transcriptase (Promega Italia Srl, Milan, Italy) and random primers (Promega) in a final volume of 20 μL. cDNAs (1 μL for each sample) were amplified by polymerase chain reaction (PCR) using the primer sequences as follows: BMP-2, 5′-CGTG TCCCCGCGTGCTTCTT-3′ (sense) and 5′-GGCTGACCTG AGTGCCTGCG-3′ (antisense); osteocalcin, 5′-CCCTCAC ACTCCTCGCCCTATT-3′ (sense) and 5′-AAGCCGATGTG GTCAGCCAACTCGT-3′ (antisense); osteonectin, 5′-AAA CCCCTCCACATT CCC-3′ (sense) and 5′-ATTTTCCGCC ACCACCTC-3′ (antisense); RUNX-2, 5′-CAC TCACTA CCACAC CTACC-3′ (sense) and 5′-TTCCATCAGCGTC AACACC-3′ (antisense). Thermal cycle parameters were as follows: 95°C for 2 min, 35 cycles of 95°C for 30 s, 52°C–60°C (depending on the T_m of each individual set of primers) for 1 min, and 72° for 30 s. GAPDH, 5′-TGGACTCCACGACG TACTCAG-3′ (sense) and 5′-ACATGTTCCAATATGATT CCA-3′ (antisense), was amplified as an internal control. The reverse transcription (RT)-PCR products were separated on 2% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet illumination.

Alizarin Red staining

Monolayers in 6-well plates (10 cm^2/well) were washed with PBS and fixed in 10% paraphormaldehyde (Sigma–Aldrich) at room temperature for 15 min. Monolayers were then washed twice with excess dH_2O before addition of 1 mL of 40 mM Alizarin Red S (pH 4.1) per well. The plates were incubated at room temperature for 20 min with gentle shaking. After aspiration of the unincorporated dye, the wells were washed four times with 4 mL dH_2O while shaking for 5 min. The plates were then left at an angle for 2 min to facilitate removal of excess water. Stained monolayers were observed by phase microscopy using an inverted microscope (Nikon). For quantification of staining, 10% acetic acid was added to each well, and the plate was incubated at room temperature for 30 min with shaking. Calcium deposits were orange-red.

Alkaline Phosphatase staining

Cells were fixed in 4% paraphormaldehyde in PBS for 20 min and then incubated for 30 min in TRIS Buffer (0.2 M, pH 8.3) with AS-MX phosphate (Sigma, St. Louis, MO) as a substrate and Fast Blue (Sigma) as a stain. The alkaline phosphatase (ALP)-positive cells stained blue/purple. Each experiment was performed in triplicate.

Results

Cell cultures

DPSCs were sorted for CD117, CD34, and STRO-1. After 30 days of culture with the standard medium plus 20% FBS, sorted cells expressed CD44, RUNX-2, and osteocalcin; had an osteoblast-like morphology with the formation of typical calcification nodules; and expressed mRNA transcripts for bone extracellular matrix proteins, as previously demonstrated by us.[6,7] On the contrary, AF cells had a heterogeneous morphology consisting primarily of two cell types: one was similar to fibroblasts, whereas the other was small and rounded, resembling epithelial cells (Fig. 1). We were able to expand AFSCs up to 2×10^6 cells within 4 weeks (3 passages) and culture them until the 30th passage. After this time, the cells became large and flat, suggestive of senescent cells.

Phenotypic characterization of AFSCs

Before coculture experiments we performed immunofluorescence and cytometric assays to confirm the stemness characteristics of AF cells. Actually, AF cells expressed all the markers commonly found on MSCs: cells were strongly positive for BMP-2, BMPR1, BMPR2, SSEA4, CD29, CD44, CD90, CD117, Tra1-60, Tra 1-80, and OCT3/4; had a weak positivity for CD133 and CD146; and were negative for CD34 and osteocalcin. Moreover, flow cytometry performed after 15 days of culture in the standard medium revealed that AFSCs expressed high levels of CD29 (98%), CD44 (94%), CD56 (92%), and CD90 (96%) and low levels of CD133 (13%), and were negative for CD31, CD34, CD45, and CD326

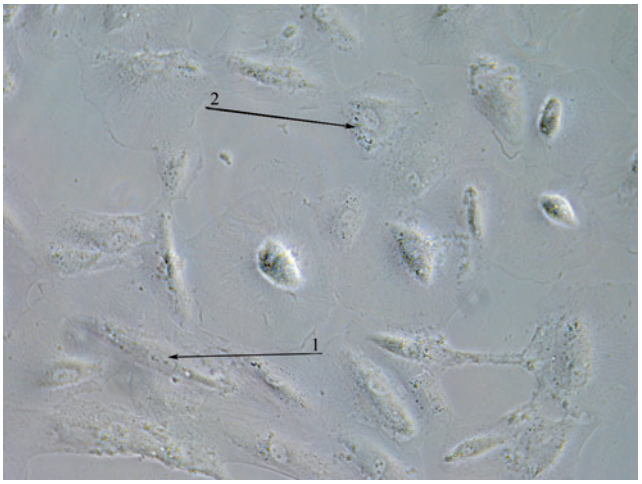

FIG. 1. AF-derived stem cell morphology consisting of two cell types: fibroblast-like (arrow 1) and small, rounded cells (arrow 2). AF, amniotic fluid. Color images available online at www.liebertonline.com/ten.

(Fig. 2). This antigenic pattern confirmed the presence of undifferentiated cells within AF, and the negativity for osteocalcin showed that an osteogenic fraction was not present within the cell population.

AFSCs cocultured with DPSC-derived osteoblasts or exposed to osteoblast-CM undergo osteogenic differentiation

After 15 days of coculture the morphology of AFSCs became homogeneous, consistently exhibiting a fibroblast-like morphology until the end of the culture period. After 10 days of coculture, AFSCs started to change their morphology and their growth. In some areas of the culture plates, AFSCs were seen to aggregate into groups, forming hemispheric centers. After 20 days, these centers formed a rounded nodular structure, and after 30 days of stimulation, calcification nodules were clearly observable (Fig. 3A). These nodules were 1–2 mm in diameter and were dark at light microscopy due to the density of the matrix (see Table 1).

Similar observations were made in the experiments using CM stimulation. After about 15 days of exposure to the CM, AFSCs started to change their morphology, building pseudo-nodular structures that at day 30 became bone (Fig. 3B).

The AFSCs cultured in an osteogenic medium (Fig. 3C) and in the same medium supplemented with VEGF and BMP-2 produced nodular structures after 3 weeks of culture. No nodular structures were observed in the plates of AFSCs grown in the standard medium. After 16 days, the cells of these cultures were confluent and remained heterogeneous,

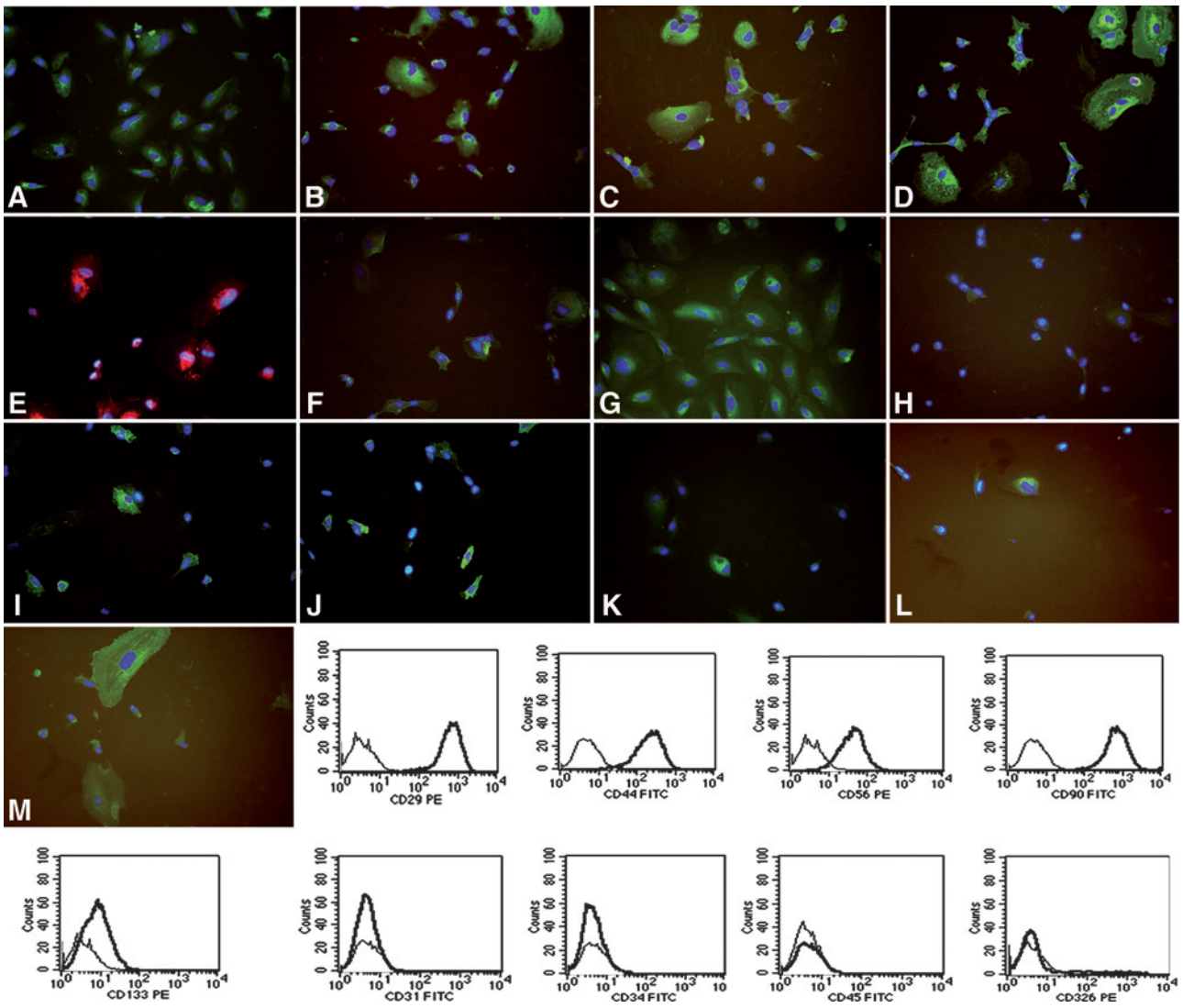

FIG. 2. Immunofluorescence analyses on AF cells for mesenchymal markers showing a strong positivity for BMP2 **(A)**, BMPR1 **(B)**, BMPR2 **(C)**, SSEA4 **(D)**, CD29 **(E)**, CD44 **(F)**, CD90 **(G)**, CD117 **(H)**, Tra1-60 **(I)**, Tra 1-80 **(J)**, and OCT3/4 **(K)**, and a weak positivity for CD133 **(L)** and CD146 **(M)** and cytometric analyses on AF-MSCs demonstrating high levels of CD29 (98%), CD44 (94%), CD56 (92%), and CD90 (96%), low levels of CD133 (13%), and negativity for CD31, CD34, CD45, and CD326. Cells are stained with FITC or PE and double-stained with 4′,6-diamidino-2-phenylindole. BMP2, bone morphogenetic protein-2; AF-MSCs, AF-derived mesenchymal stem cells. Color images available online at www.liebertonline.com/ten.

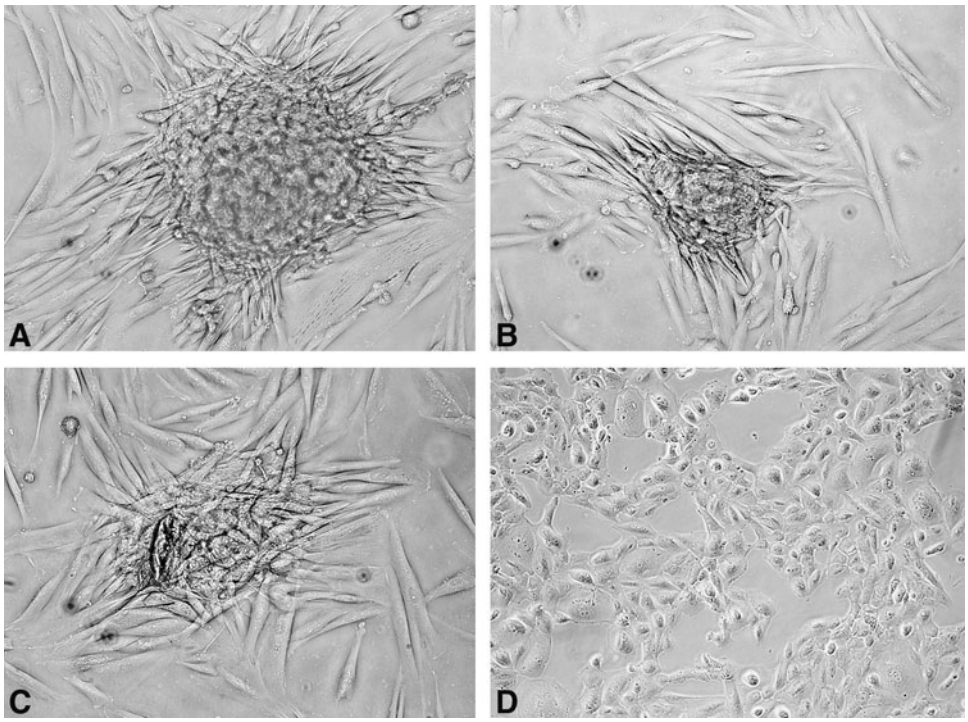

FIG. 3. Calcification nodules in **(A)** AF-MSCs/DPSCs cocultures; **(B)** in AF-MSCs cultured in the conditioned medium; **(C)** in AF-MSCs cultured in the osteogenic medium; **(D)** no nodular structures were found in AF-MSCs cultured in the standard medium, after 30 days of culture. DPSCs, dental pulp stem cells.

with a round, fibroblast-like morphology, growing more slowly compared to initial passages of cultures (Fig. 3D).

BMP-2 and VEGF quantitative detection

Enzyme-linked immunosorbent assays were performed at 24, 48, 72, and 96 h after plating to measure BMP-2 and VEGF levels in the medium of $OC^+/RUNX^+$ DPSCs. We found that $OC^+/RUNX^+$ DPSCs secreted high levels of BMP-2 and VEGF, which increased with time. The level of VEGF was 145.9 ± 7 pg/mL at 24 h and 411 ± 13 pg/mL at 96 h ($p < 0.001$) (Fig. 4A), whereas that of BMP-2 was 113.8 ± 15 pg/mL at 24 h and 193.4 ± 20 pg/mL at 96 h ($p < 0.001$), (Fig. 4B).

RT-PCR, Alizarin Red S, and ALP analyses

At 15 and 30 days of coculture, RT-PCR analyses revealed that AFSCs cocultured with $OC^+/RUNX\text{-}2^+$ cells were able to differentiate into osteoblasts because of expression of genes encoding for osteogenic markers such as BMP-2, RUNX-2, osteocalcin, and osteonectin. The same results were obtained in AFSCs stimulated with the CM of $OC^+/RUNX\text{-}2^+$ DPSCs and in AFSCs cultured in the osteogenic medium. Interestingly, cells cultured in the osteogenic medium supplemented with VEGF and BMP-2 showed a significant RUNX-2 gene upregulation. Cells cultured in only the standard medium also expressed genes encoding for BMP-2 and osteonectin. Moreover, they expressed high levels of mRNA transcript of RUNX-2, but did not express mRNA transcript for osteocalcin (Fig. 5A).

To confirm osteogenic differentiation of AFSCs cocultured with $OC^+/RUNX\text{-}2^+$ DPSCs and AFSCs cultured with the CM of $OC^+/RUNX\text{-}2^+$ DPSCs, Alizarin Red S and ALP staining were performed. Alizarin Red S reveals calcium accumulation within cells and ALP is an enzyme required for mineral deposition. Cells from cocultures and those stimulated with the CM showed Alizarin Red–positive condensed nodules with high levels of calcium deposits; no staining was seen in the control cultures grown in the standard medium (Fig. 5B). ALP staining was clearly observable in bone nodules and the surroundings cells, confirming their nature as mineralization and ossification centers both in cocultures and in CM experiments. AFSCs grown in an osteogenic medium were positive for Alizarin Red S and ALP (Fig. 5C).

Table 1. Number of Bone Nodules and Percentages of Bone Differentiation in All Culture Conditions

	No. of calcification nodules		*Percentage of bone differention*	
Culture conditions	*15 days*	*30 days*	*15 days*	*30 days*
AF-MSCs standar medium	0	0	0	0
AF-MSCs osteogenic medium	3	21	12	75
AF-MSCs osteogenic medium + bone morphogenetic protein-2 + vascular endothelial growth factor	7	20	17	77
AF-MSCs conditioned medium	15	32	41	91
AF-MSCs/dental pulp stem cells coculture	17	35	48	98

AF-MSCs, amniotic fluid-derived mesenchymal stem cells.

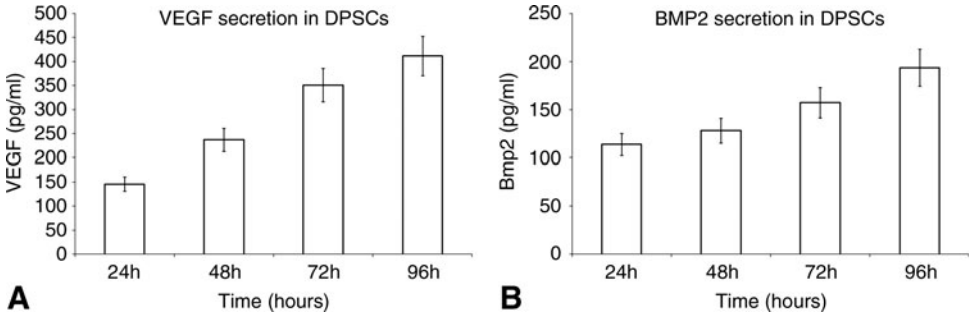

FIG. 4. Enzyme-linked immunosorbent assays performed for **(A)** VEGF and **(B)** BMP-2 released in the medium by $OC^+/Runx^+$ DPSCs after 24, 48, 72, and 96 h after plating. VEGF, vascular endothelial growth factor.

Discussion

There is growing evidence on the presence of MSCs in many tissues of the human body. AF is known to contain multiple cell types derived from the developing fetus.[17,18] Cells within this heterogeneous population can give rise to a variety of differentiated cells, including those of adipose, muscle, bone, and neuronal lineages.[10,13,14] In this study, we have demonstrated that AF progenitors can commit to the osteogenic lineage when exposed to $RUNX\text{-}2^+/OC^+$ cells

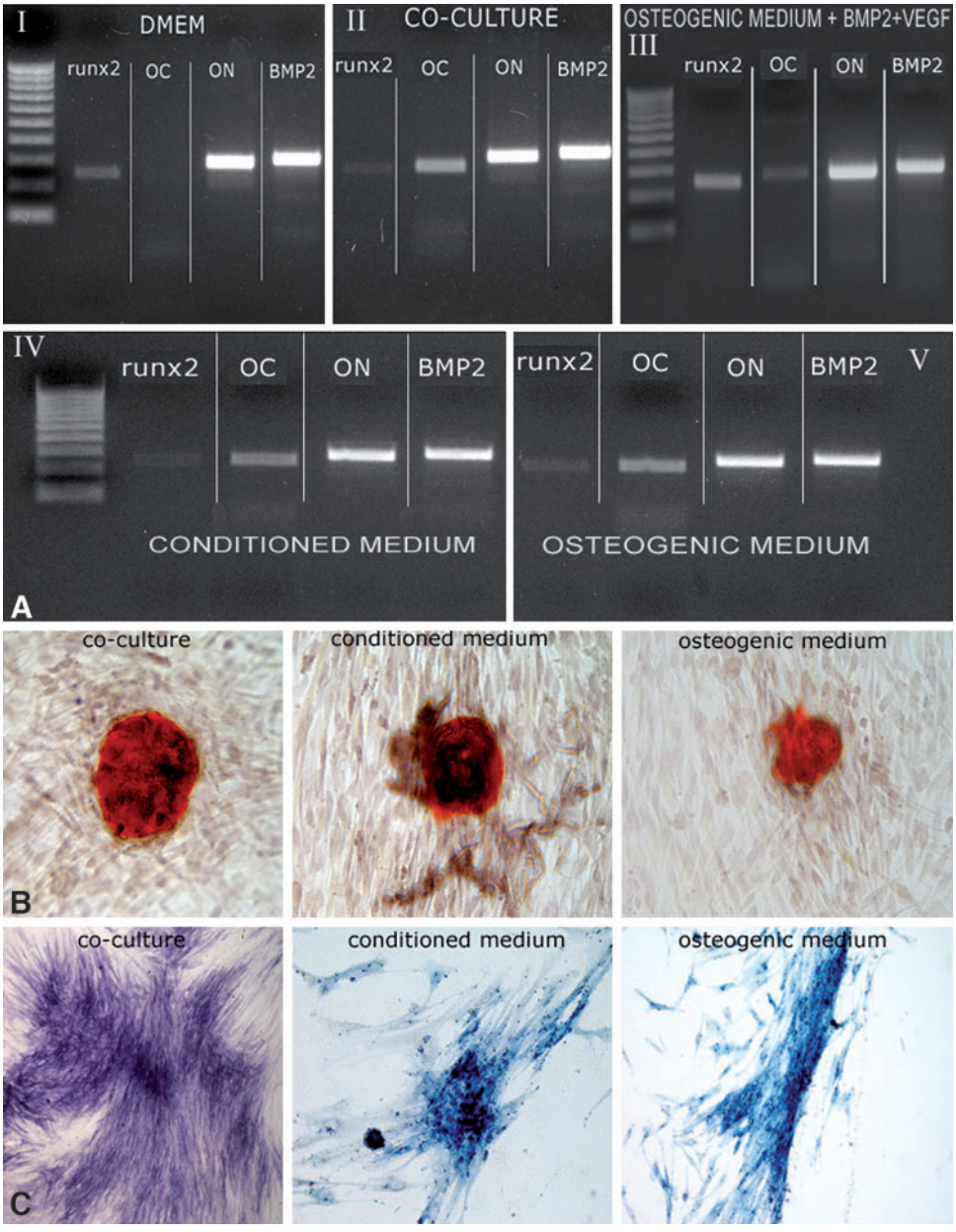

FIG. 5. **(A)** RT-polymerase chain reaction analyses for Runx-2, osteocalcin (OC), osteonectin (ON), and BMP-2, in all culture conditions (I, AF-MSCs grown in Dulbecco's modified Eagle's medium; II, cocultured; III, osteogenic medium added with BMP-2 and VEGF; IV, conditioned medium; V, osteogenic medium) showing osteogenic differentiation of AF-MSCs cocultured with $OC^+/Runx\text{-}2^+$ cells and stimulated with the conditioned medium, as well as Runx-2 upregulation by VEGF and BMP-2; **(B)** Alizarin Red S and **(C)** alkaline phosphatase staining for AF-MSCs, in all culture conditions, showing the osteogenic differentiation of AF-MSCs. Color images available online at www.liebertonline.com/ten.

(obtained from the differentiation of human DPSCs in our case), and when AFSCs were cultured with the CM of RUNX-2^+/OC^+ DPSCs. DPSCs are bone-committed cells and form a good model of osteogenic differentiation that can be used to study the regenerative mechanisms of the bone.[6–8,16,19] The ability to obtain osteogenic differentiation and the production of a well-differentiated bone matrix by culturing the cells with OC^+/RUNX-2^+ cells and using the CM of RUNX-2^+/OC^+ DPSCs is very important in the perspective of clinical applications of these cells. Although DPSCs are multipotent stem cells,[6,20] their self-commitment toward an osteogenic fate mimics the bone regeneration occurring after injury. Indeed, this *in vitro* model has been shown to be helpful in the study of the bone development processes.[16,21] In this study, we used OC^+/RUNX-2^+ differentiated stem cells instead of bone cell lineages to mimic the physiology of bone differentiation microenvironment in terms of soluble factors released during the differentiation process.

Before the coculture and CM stimulation experiments, we performed immunofluorescence and cytometric assays on AFSCs to evaluate their stemness and possible expression of osteogenic/endothelial/hematopoietic and epithelial differentiation markers. AFSCs are positive for CD29, CD44, CD56, CD90, CD117, CD146, SSEA4, and OCT3/4, highlighting that they are MSCs with expression of several early embryonic/fetal cells. In particular, $>90\%$ of the AF cells expressed the transcription factor OCT-3/4, which is associated with maintenance of the undifferentiated state and pluripotency of embryonic stem cells. CD117 is the receptor of stem cell factor, mainly expressed on hematopoietic and mesenchymal cells. Its expression together with OCT3/4, CD29, CD56, CD90, and SSEA4 is a clear indication of MSCs stemness. Moreover, AF cells expressed characteristic markers of embryonic germ cells and embryonic stem cells, including Tra1-60 and Tra 1-80. Interestingly, AF cells constitutively expressed BMP-2 and both receptors BMPRI and BMPRII. This is likely due to an intrinsic plasticity addressed toward an osteogenic lineage. Finally, these cells were also positive, although to a lesser extent, for CD133, which is another stemness marker, mainly expressed on neuronal, epithelial, and MSCs.

The absence of osteocalcin underlines that no osteogenic fraction/subsets were present within the tested cell population; in addition, CD31, CD34, and CD45 negativity indicates that AFSCs do not belong to the hematopoietic and endothelial lineages. Moreover, AFSCs were negative for CD326 (also termed EpCAM), an epithelial lineage marker, confirming thus their mesenchymal origin.

Taking all these findings into consideration, we performed cocultures to evaluate the effects of OC^+/RUNX-2^+ DPSCs on AFSC differentiation. After 20 days of coculture, we observed that AFSCs first formed aggregates and then developed hemispheric ossification centers that grew into rounded structures that were macroscopically detectable. At the same time, we cultured AFSCs in the CM derived from OC^+/RUNX-2^+ DPSCs to evaluate the effect of soluble factors produced by OC^+/RUNX-2^+ DPSCs on osteogenic differentiation of AFSCs. After 30 days of exposure to the CM, calcification nodules were observed in plates as observed by light microscopy.

To further demonstrate the ability of DPSCs to differentiate into bone, we performed RT-PCR and Alizarin Red S and ALP staining after 15 and 30 days coculture or CM stimulation. The appearance of a mineralized matrix was clearly observed both with Alizarin Red and ALP staining, which specifically stains calcium deposits, demonstrating that AFSCs were able to differentiate into osteoblasts. This was confirmed by RT-PCR for BMP-2, osteocalcin, osteonectin, and RUNX-2 transcripts. Interestingly, we detected high levels of RUNX-2 and low levels of osteonectin in cultures in which AFSCs grew in a standard medium, showing that RUNX-2 and osteonectin themselves were not able to effectively induce bone differentiation. Only AFSCs were capable of achieving an effective osteogenic fate, as evidenced by the positivity for osteocalcin, which is a marker of terminal osteoblast differentiation and bone formation. Further, AFSCs grown in a standard medium, although expressing genes encoding for osteogenic markers, did not produce nodular structures during the culture period, maintained a heterogeneous fibroblast-like morphology, and were not stained by Alizarin Red S.

RUNX-2 is a transcription factor involved in osteogenic differentiation and its expression decreases during terminal osteoblast differentiation. In the cocultures, RUNX-2 levels decreased when compared to cells cultured alone. This finding, together with osteocalcin expression, indicates that osteogenic differentiation takes place and suggests different roles of these genes in the regulation of bone formation. In this context, it is very important to consider the role of soluble factors released from DPSCs into the coculture medium, CM, and osteogenic medium supplemented with VEGF and BMP-2 that might induce osteogenic differentiation of AFSCs. There is evidence demonstrating that several soluble proteins extracted from human teeth regulate odontogenesis and mineral formation,[22,23] such as BMPs, enamelin, decorin, bone sialoprotein, and fibronectin. In this context, we evaluated both the BMP-2 and VEGF levels in OC^+/RUNX-2^+ DPSCs medium and the effect of BMP-2 and VEGF on osteogenic differentiation. BMP and VEGF are two important factors for osteogenesis. BMP-2 is a member of the transforming growth factor superfamily and plays an essential role during skeletal development and growth. BMP-2 is also used widely for therapeutic bone applications. VEGF is a heparin-binding glycoprotein with strong angiogenic and mitogenic activities.[24–26] VEGF is an endothelial cell mitogen *in vitro*, while *in vivo* acts as a chemotactic factor for monocytes and osteoblasts can induce angiogenesis and increase microvascular permeability.[27] In addition, VEGF is of critical importance for bone development as vessel formation is directly linked to bone differentiation.[28] By their involvement in osteogenic processes and angiogenesis, these factors can influence the biological responses and bone differentiation programme.

In this study, BMP-2 and VEGF released into the medium by OC^+/RUNX-2^+ DPSCs may be responsible for the bone differentiation of AFSCs. In our experiments, AFSCs, when cultured as reported above, seem to be able to induce bone differentiation and they may provide an excellent alternative source of human MSCs with potential therapeutic applications. Interestingly, VEGF and BMP-2 morphogens, when added to the osteogenic medium, were able to promote osteoblastic differentiation/maturation by upregulating expression of RUNX-2, as confirmed by RT-PCR. Further investigations are needed to fully assess this

possibility. On the other hand, DPSCs have already been used successfully with titanium implants[20] for several applications in humans, such as bone defect repair.[19] Hopefully, MSCs may be used in the near future for heterologous transplants, due to their unique outstanding features.[29,30] In this view, the ability of osteoblasts that have been differentiated from DPSCs to induce AFSCs to form bone is a further possibility that can be taken into consideration for clinical applications.

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Disclosure Statement

All authors have no conflicts of interest.

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Address correspondence to:
Gianpaolo Papaccio, M.D., Ph.D.
Dipartimento di Medicina Sperimentale
Sezione di Istologia ed Embriologia
Secondo Ateneo di Napoli
5, via L. Armanni
80138 Napoli
Italia

E-mail: gianpaolo.papaccio@unina2.it

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